

# Changes in HER2 Expression and Amplification Status Following Preoperative Chemotherapy for Gastric Cancer

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**Abstract.** *Background: It is essential to establish a strategy for second-line treatment for human epidermal growth factor receptor 2 (HER2)-positive gastric cancer; however, HER2 expression status after chemotherapy treatment is not routinely determined. Materials and Methods: We analyzed 25 cases of gastric cancer that received preoperative chemotherapy and selected the six pre-treatment samples that were HER2-positive. Pre- and post-treatment tumor samples were examined for HER2 expression, and for HER2, epidermal growth factor receptor (EGFR), and hepatocyte growth factor receptor (MET) gene amplification. Results: Three patients had been treated with trastuzumab plus chemotherapy, and three patients with cytotoxic chemotherapy alone. Only one case that had an initial HER2 score of 3+ and had received trastuzumab plus chemotherapy remained HER2-positive after treatment. Decrease or loss of HER2 expression and amplification was observed in the other five patients. Amplification of EGFR or MET was not observed in any pre- or post-treatment specimens. Conclusion: Our data suggest that trastuzumab plus chemotherapy or chemotherapy alone may induce loss of HER2 positivity.*

Approximately 10–30% of gastric cancer cases are human epidermal growth factor receptor 2 (HER2)-positive and are possible targets for anti-HER2 therapy (1-5). The phase III Trastuzumab for Gastric Cancer (ToGA) study was the first trial to demonstrate a significant therapeutic benefit of

trastuzumab, a humanized monoclonal antibody to HER2, in combination with chemotherapy against HER2-positive gastric or gastro-esophageal junction cancer (6). Regarding second-line treatment, the efficacy of continuous anti-HER2-targeted therapy has been investigated. In the TyTAN trial, which explored the efficacy of lapatinib for the second-line treatment of HER2-positive advanced gastric cancer, the addition of lapatinib to second-line paclitaxel was not superior compared to placebo plus paclitaxel (7). In the GATSBY trial, trastuzumab emtansine (T-DM1) was not superior to taxane monotherapy in patients with previously treated HER2-positive gastric or gastro-esophageal junction cancer (8). It is also noteworthy that in the GATSBY trial, T-DM1 failed to prove its superiority over taxane in patients who had received cytotoxic therapy alone (23%) and in those who had been previously treated with HER2-targeted therapy (77%) (8).

Mechanisms to explain these disappointing results have been proposed. One explanation is that HER2 positivity is lost after HER2-targeted treatment. In breast and gastric cancer, it has been reported that previously treated tumors may lose HER2 expression after HER2-targeted therapy (9-13). The selective pressure of HER2-targeted treatment has been proposed as one of the mechanisms whereby HER2 expression is lost. Since trastuzumab exerts its antitumor effects against HER2-positive tumor cells (14, 15), it may preferentially eradicate HER2-overexpressing cells, resulting in the selective survival of HER2-negative tumor cells. In addition, gastric cancer has been reported to have greater heterogeneity of HER2 expression than breast cancer (16, 17). Treatment-induced change in HER2 status may occur more frequently in gastric cancer because HER2-negative tumor cells would become the dominant population in tumors after HER2-targeted therapy. Expression of other receptor tyrosine kinases (RTKs) might be another mechanism that could drive resistance to molecularly targeted therapy through proliferation of non-targeted tumor

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cells after treatment (18). Tumors might either initially co-express multiple RTKs or shift their proliferative dependency onto other RTKs following molecularly-targeted therapy. Indeed, it has been reported that gastric cancer may co-express HER2, epidermal growth factor receptor (EGFR), and hepatocyte growth factor receptor (MET) (19, 20).

Although several mechanisms have been proposed to explain the results of second-line HER2-targeted therapy in gastric cancer, the reason why HER2-targeted therapy has not shown clinical advantage even in patients not treated with HER2-targeted therapy remains elusive. In this study, we focused on patients with gastric cancer who received preoperative chemotherapy and aimed to examine the changes in HER2 expression status and amplification of *EGFR* and *MET*, not only after HER2-targeted therapy, but also after cytotoxic chemotherapy alone.

## Materials and Methods

**Patients.** Twenty-five patients with gastric cancer who received preoperative chemotherapy between 2009 and 2015 at the Department of Surgery and Science, Kyushu University Hospital were analyzed. Patients who received neoadjuvant chemotherapy for a resectable tumor and who were converted to surgical resection after chemotherapy were included. Two patients enrolled in a clinical trial were also included in this study. Informed consent was obtained from all patients. The local Ethics Committees of Kyushu University (Study number, 28-68) and Chugai Pharmaceutical Co., Ltd. (Study number, E181) approved the study.

**Immunohistochemical staining of HER2.** Formalin-fixed, paraffin-embedded pre- and post-treatment tumor samples were examined for HER2 expression using immunohistochemistry (IHC). After deparaffinization, sections were treated with Target Retrieval Solution (pH 6.0; Dako, Agilent, Santa Clara, CA, USA) in a microwave at 95°C for 40 min. Slides were then cooled for 30 min at room temperature and treated with methanol containing 3% H<sub>2</sub>O<sub>2</sub> to block endogenous peroxidase activity. After incubation with 10% goat serum for 10 min, slides were incubated with an antibody to HER2 (A0485; Dako) at 1:400 dilution overnight at 4°C, and incubated with horseradish peroxidase polymer-conjugated secondary antibodies (Dako) for 1 h. Sections were then color-developed with 3, 3'-diaminobenzidine, counterstained with 10% Mayer's hematoxylin, dehydrated, and mounted. HER2 expression was scored according to previously described scoring criteria (21-23) as follows: Score of 0, no staining or membranous staining in <10% of tumor cells (surgical specimen) or fewer than five cohesive tumor cells (biopsy specimen); score of 1+, weak or detectable staining in only one part of the membrane in ≥10% of tumor cells (surgical specimen) or at least five cohesive tumor cells (biopsy specimen); score of 2+, weak to moderate complete or basolateral membranous staining in ≥10% of tumor cells (surgical specimen) or at least five cohesive tumor cells (biopsy specimen); score of 3+, moderate to strong complete or basolateral membranous staining in ≥10% of tumor cells (surgical specimen) or at least five cohesive tumor cells (biopsy specimen).

**Multicolor fluorescence in situ hybridization (FISH) of *EGFR*, *MET*, and *HER2*.** Formalin-fixed, paraffin-embedded tumor samples were

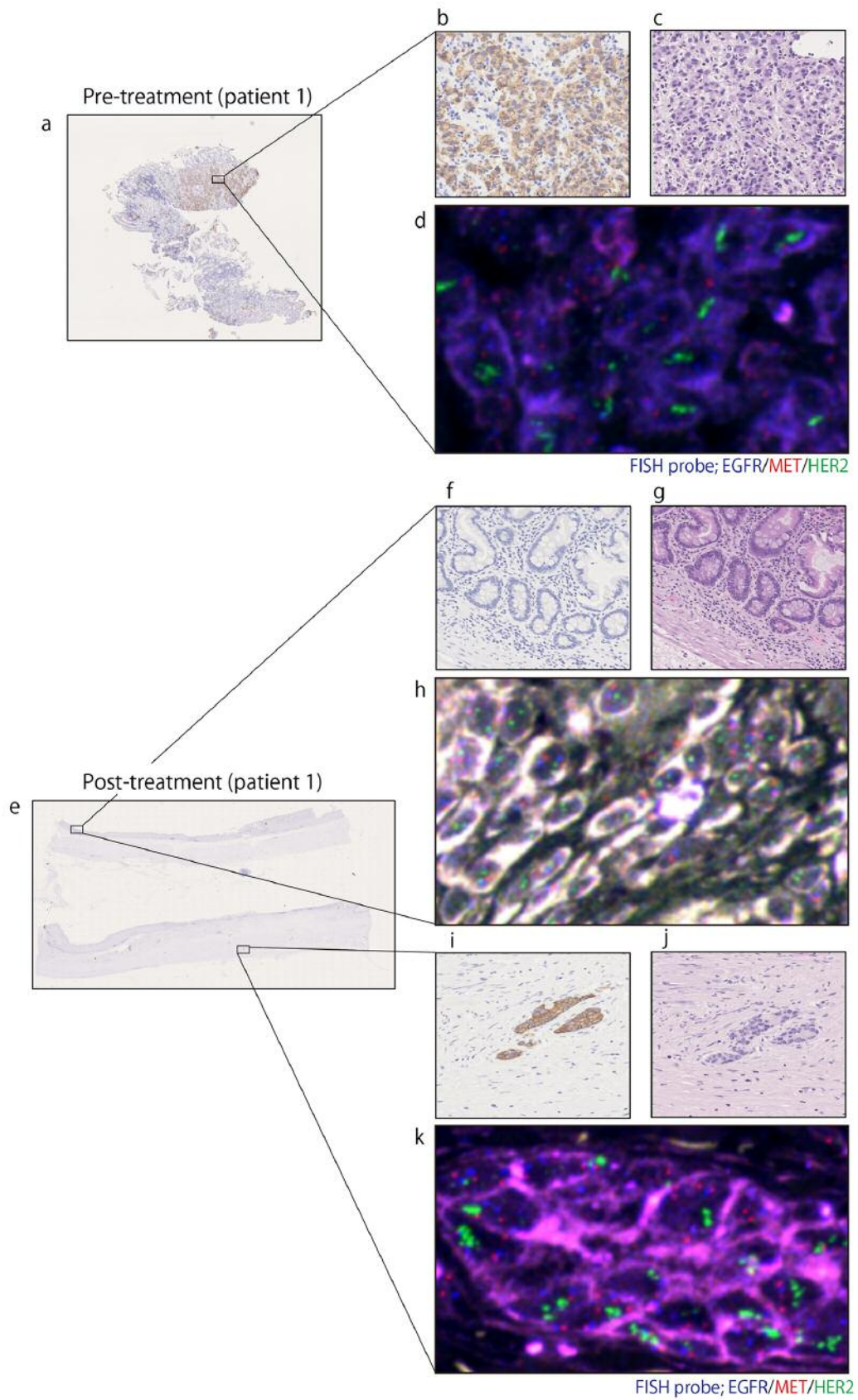
examined for *HER2*, *EGFR* and *MET* amplification using FISH. A multicolor FISH probe [*EGFR* (Cy 5.5)/*MET* (TexRed)/*HER2* (fluorescein isothiocyanate)] was constructed by GSP Laboratory (Kobe, Japan). FISH analysis was performed using pretreatment kit II (GSP Laboratory) according to the manufacturer's instructions. In cases where multicolor FISH signals were faint, samples were re-analyzed using the following dual color FISH probes: *HER2/CEN17*, *EGFR/CEN7*, and *MET/CEN7* (GSP Laboratory). In all cases, at least 20 cells from more than three different regions of a specimen were examined. HER2-amplified cases were defined as those with an average HER2 gene copy number ≥6 signals per cell or the presence of gene clusters (24). *EGFR*- or *MET*-amplified cases were defined as these with gene copy number ≥6 signals per cell in ≥40% of cells, ≥15 signals per cell in ≥10% of cells, or the presence of gene clusters in ≥10% of cells (25). Fluorescence image acquisition was performed using a Nikon A1R confocal imaging system (Nikon Corporation, Tokyo, Japan). The objective lens was an oil immersion Plan-Apo ×60 numerical aperture 1.40 lens (Nikon).

## Results

**HER2 expression and amplification in patients with gastric cancer who had received preoperative chemotherapy.** In 25 patients with gastric cancer who had received preoperative chemotherapy, there were five patients with preoperative biopsy samples that were HER2 3+ and one patient who was HER2 2+ with *HER2* FISH amplification, and thus considered HER2-positive. In order to examine changes in HER2 status after chemotherapy, we assessed the surgical specimens of these six cases of HER2 expression, and also performed FISH for *EGFR*/*MET*/*HER2* amplification in pre- and post-treatment specimens. As shown in Table I, all these specimens had *HER2* amplification in the concordant region of the tumor (Figures 1 and 2). Neither *EGFR* nor *MET* were amplified in any of their pre- and post-treatment specimens.

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Figure 1. Immunohistochemical (IHC) staining of human epidermal growth factor receptor 2 (HER2), and fluorescence in situ hybridization (FISH) of epidermal growth factor receptor (EGFR)/hepatocyte growth factor receptor (MET)/HER2 in a case that received trastuzumab in combination with preoperative chemotherapy (patient 1 in Table I). a-d: Pre-treatment biopsy specimen. e-k: Post-treatment surgical resected specimen from the same patient. a, b, e, f, i: HER2 IHC staining (magnification: a; ×3, b, f, i; ×60, e; ×2.5). c, g, j: Hematoxylin and eosin staining (magnification: ×60). d, h, k: FISH signals of *EGFR*/*MET*/*HER2* (magnification: ×500). FISH was performed with a probe for *EGFR*/*MET*/*HER2* in the same region as HER2 IHC staining. *EGFR*, *MET* and *HER2* gene signals are represented as blue, red, and green, respectively. HER2 IHC score was 3+ in pre- and post-treatment specimens. HER2 amplification was observed in the same region as HER2 IHC staining, whereas no *EGFR* or *MET* amplification was observed.



*Changes in HER2 expression and amplification after preoperative chemotherapy.* In the six patients who were initially HER2-positive, three patients were treated with trastuzumab in combination with chemotherapy; the other three patients were treated with cytotoxic chemotherapy alone, either S-1, docetaxel, or cisplatin (Table I). The details of the therapy regimens used are also summarized in Table I. Patient 1 treated with trastuzumab in combination with chemotherapy had an initial HER2 IHC score of 3+ and HER2 gene amplification. After treatment, HER2 expression and amplification were retained in the surgical specimen from the same patient (Table I; Figure 1). In the post-treatment specimen of this case, HER2-positive tumor cells were observed in the mid-portion of the tissue and HER2-negative tumor cells were seen in the epithelial portion (Figure 1). Two other patients who had received trastuzumab in combination with chemotherapy showed a decrease or loss of HER2 expression and amplification in post-treatment surgical specimens (patients 2 and 3 in Table I). We also found that in all three cases treated with cytotoxic chemotherapy alone, HER2 expression was lost (IHC score 0) after treatment (Figure 2, patients 4-6 in Table I). In their post-treatment specimens, loss of *HER2* gene amplification was also confirmed in all three cases using FISH. Results are summarized in Table I.

## Discussion

Since trastuzumab demonstrated a significant overall survival advantage as first-line therapy for advanced HER2-positive gastric cancer (6), the HER2 signaling pathway is considered to be a driver pathway of proliferation in HER2-positive gastric tumor cells. Although HER2-targeted therapy has been commonly used in second-line treatment of HER2-positive breast cancer (26), it has failed to be established as second-line treatment in gastric cancer. Consistent with previous studies in gastric cancer (11-13), we found that two out of three patients with HER2-positive disease treated with trastuzumab in combination with chemotherapy were converted to HER2-negative. In addition, our data suggest that cytotoxic chemotherapy can also lead to loss of HER2-positive tumor cells. The loss of HER2 was confirmed by both protein expression and gene amplification analyses, suggesting that the loss of HER2 expression observed was not due to inappropriate IHC staining conditions that would affect IHC scores (27).

In breast cancer, Guarneri *et al.* reported that HER2 loss was observed more frequently after cytotoxic chemotherapy alone than after chemotherapy in combination with anti-HER2 targeted therapy (40% versus 14.7%) (9). Although the reason for this phenomenon observed in the current study and in the breast cancer study is unclear, several preclinical studies showed that HER2 overexpression would promote proliferation of tumor cells (28, 29). Therefore, one possible

explanation for cytotoxic chemotherapy-induced loss of HER2 is that HER2-positive gastric cancer cells may proliferate faster than other tumor cells, therefore, they would be more sensitive to cytotoxic therapies. Indeed, several clinical studies support the hypothesis that HER2-positive tumors are more sensitive to chemotherapy (30, 31), suggesting that, like HER2-targeted therapy, cytotoxic therapy may also preferentially eliminate HER2-positive clones, resulting in loss of HER2 positivity. This finding may provide a possible explanation for the result of the GATSBY trial, in which T-DM1 did not show superior clinical benefit compared with taxane in patients who were previously treated with or without HER2-targeted therapy.

Because it has been shown that molecular heterogeneity and receptor co-amplification can drive resistance to targeted therapies (18), we investigated whether the tumors had co-amplification of *EGFR* or *MET* and whether amplification would be affected by chemotherapy. In our data, neither *EGFR* nor *MET* were amplified in pre- and post-treatment specimens, suggesting that *EGFR*- or *MET*-amplified tumor cells did not pre-exist or arise during chemotherapy treatment in these cases. However, we cannot rule out the involvement of other RTKs or downstream signaling factors that were previously reported to be amplified or overexpressed in gastric cancer (32, 33).

Our work has certain limitations. Firstly, since our results are based on a small sample size, it is difficult to estimate the rate of change of HER2 expression after chemotherapy statistically. Secondly, discordance of HER2 expression between biopsy and surgical specimens may also have influenced the results (34, 35). Biopsy specimens may fail to reflect HER2 expression in the whole tumor. Thus, there is still the possibility that we overestimated HER2 expression in pre-treatment specimens. Therefore, further comprehensive assessments are warranted.

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Figure 2. Immunohistochemical (IHC) staining of human epidermal growth factor receptor 2 (HER2), and fluorescence in situ hybridization (FISH) of epidermal growth factor receptor (EGFR)/hepatocyte growth factor receptor (MET)/HER2 in a case that received cytotoxic preoperative chemotherapy (patient 6 in Table I). a-g: Pre-treatment biopsy specimen. h-k: Post-treatment surgical resected specimen from the same patient. a, b, e, h, i: HER2 IHC staining (magnification: a:  $\times 3$ , b, e, h, i:  $\times 100$ , h:  $\times 2.5$ ). c, f, j: Hematoxylin and eosin staining (magnification:  $\times 100$ ). d, g, k: FISH signals of EGFR/MET/HER2 (magnification:  $\times 500$ ). FISH was performed with a probe for EGFR/MET/HER2 in the same region as HER2 IHC staining. EGFR, MET and HER2 gene signals are represented as blue, red, and green, respectively. HER2 IHC score was 3+ in the pre-treatment specimen and 0 in the post-treatment specimen. HER2 amplification was observed in the same region as HER2 IHC staining, whereas no EGFR or MET amplification was observed.



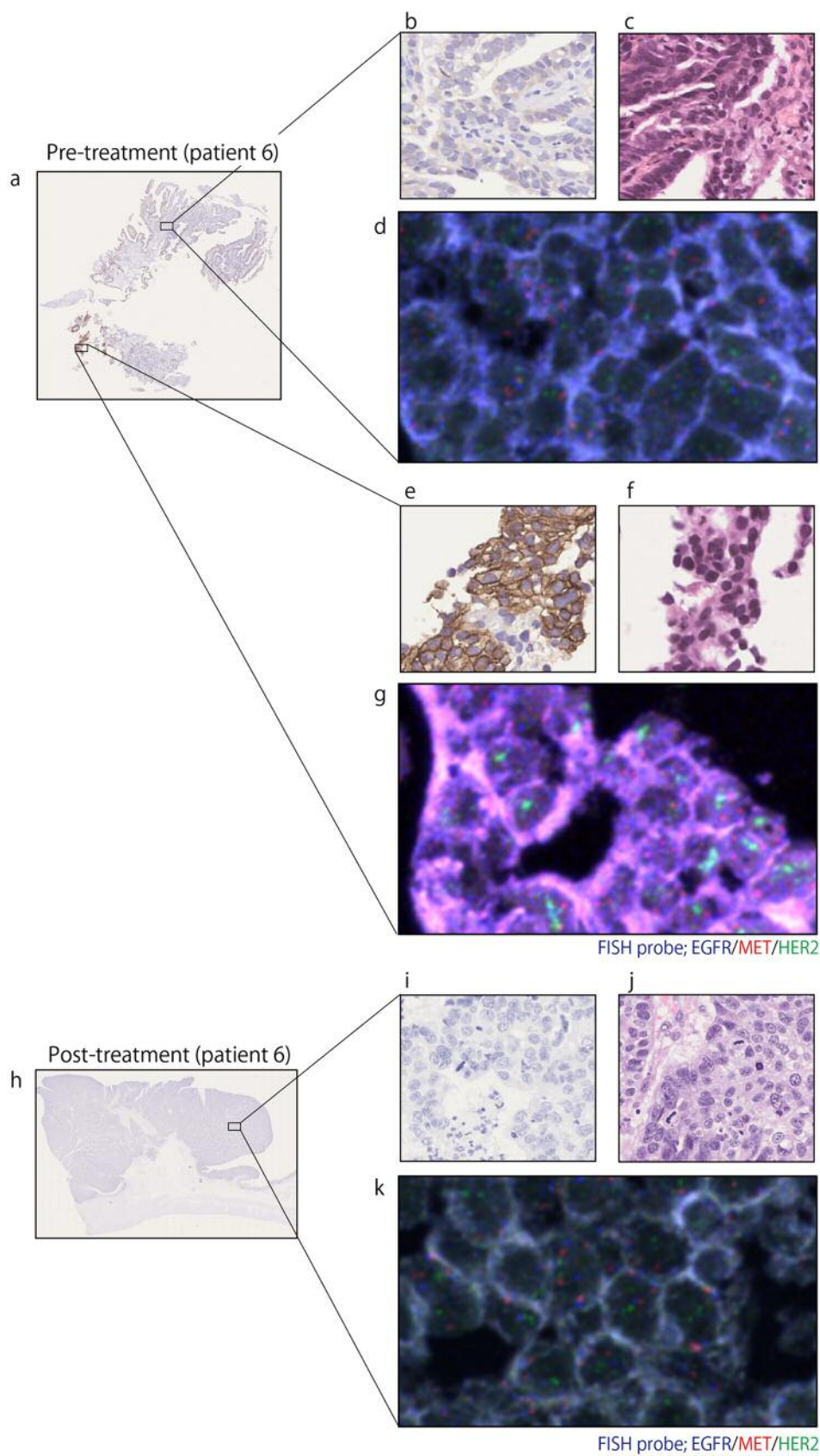


Table I. Changes in human epidermal growth factor receptor 2 (HER2) immunohistochemistry (IHC) score and amplification status after preoperative chemotherapy.

Treatment regimens										Pre-treatment			Post-treatment				
Patient	Gender	Age (Years)	Stage pre-treatment	Initial dosage of treatment	Duration	Initial dosage of treatment	Duration	Clinical response (Grade)	HER2 IHC score	Amplification			Amplification				
										HER2	EGFR	MET	HER2	EGFR	MET	HER2 IHC score	HER2
Trastuzumab	1	M	38	IV	120 mg/day, S-1 60 mg/m <sup>2</sup> , cisplatin	5 Weeks × 1	8 mg/kg, trastuzumab 3,000 mg/day, capecitabine 80 mg/m <sup>2</sup> , cisplatin	3 Weeks × 1	2	3	+	-	-	3	+	-	-
	2	M	60	IV	8 mg/kg, trastuzumab 3,600 mg/day, capecitabine 80 mg/m <sup>2</sup> , cisplatin Investigational agent or placebo <sup>‡</sup>	3 Weeks × 4		1	2	+	-	-	1	-	-	-	
	3	M	64	IV	8 mg/kg, trastuzumab 3,600 mg/day, capecitabine 80 mg/m <sup>2</sup> , cisplatin Investigational agent or placebo <sup>‡</sup>	3 Weeks × 5		2	3	+	-	-	0	-	-	-	
No trastuzumab	4	M	82	IV	100 mg/day, S-1 120 mg/day, S-1 35 mg/m <sup>2</sup> , docetaxel	3 Weeks × 6		1a	3	+	-	-	0	-	-	-	
	5	F	58	IIIB	120 mg/day, S-1 35 mg/m <sup>2</sup> , docetaxel	4 Weeks × 3		2	3	+	-	-	0	-	-	-	
	6	M	76	IV	120 mg/day, S-1 60 mg/m <sup>2</sup> , cisplatin	5 Weeks × 2		1b	3	+	-	-	0	-	-	-	

M: Male, F: female. EGFR: epidermal growth factor receptor; MET: hepatocyte growth factor receptor; <sup>‡</sup>Participants of a clinical study.

In conclusion, we assessed HER2 status after preoperative chemotherapy and found that cytotoxic chemotherapy alone could lead to loss of HER2 expression and gene amplification. Our data suggest that even in cases that have not previously received HER2-targeted therapy, it is critically important to reassess HER2 expression prior to treatment with HER2-targeted therapy. In order to accurately evaluate efficacy of second-line HER2-targeted therapy in gastric cancer, future clinical trials need to be designed, such that only patients who remain HER2-positive after previous chemotherapy treatment are eligible.

## Conflicts of Interest

S. Shu is an employee of Chugai Pharmaceutical Co. Ltd., M. Iimori is a staff member of an endowed course at Kyushu University funded by a donation from Taiho Pharmaceutical Co., Ltd. All other Authors have no conflicts of interest to declare.

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